

## Nafamostat Mesilate: Can It Be Used as a Conduit Preserving Agent in Coronary Artery Bypass Surgery?

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**Background:** Graft vessel preservation solution in coronary artery bypass surgery is used to maintain the graft conduit in optimal condition during the perioperative period. Nafamostat mesilate (NM) has anticoagulation and anti-inflammatory properties. Therefore, we investigated NM as a conduit preservative agent and compared it to papaverine. **Methods:** Sprague-Dawley (SD) rat thoracic aortas were examined for their contraction-relaxation ability using phenylephrine (PE) and acetylcholine (ACh) following preincubation with papaverine and NM in standard classical organ baths. Human umbilical vein endothelial cells (HUVECs) were cultured to check for the endothelial cell viability. Histopathological examination and terminal deoxynucleotidyl transferase dUTP nick end labeling assay were performed on the thoracic aortas of SD rats. **Results:** The anti-contraction effects of papaverine were superior to those of NM at PE ( $p < 0.05$ ). The relaxation effect of NM on ACh-induced vasodilatation was not statistically different from that of papaverine. Viability assays using HUVECs showed endothelial cell survival rates of  $>90\%$  in various concentrations of both NM and papaverine. A histopathological study showed a protective effect against necrosis and apoptosis ( $p < 0.05$ ) in the NM group. **Conclusion:** NM exhibited good vascular relaxation and a reasonable anti-vasocontraction effect with a better cell protecting effect than papaverine; therefore, we concluded that NM is a good potential conduit preserving agent.

Key words: 1. Coronary artery bypass surgery  
2. Coronary artery bypass conduits  
3. Anticoagulants

### INTRODUCTION

Coronary artery bypass surgery (CABG) is effective in coronary artery diseases that are not effective by intervention [1]. CABG is known to be particularly effective for treating high-risk group patients who may have left main coronary ar-

tery disorders, severe ventricular functional defects, or diabetic conditions [2,3].

Preservative solution for a graft conduit in a CABG procedure is used mainly in a mixture with antispastics and anticoagulants. The antispastic drug prevents the contraction of blood vessels, and the anticoagulant drug prevents the for-

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mation of blood clots. A component that maintains high viability of vascular cells is also added to the preservative solution for a graft conduit before the solution is used in CABG [4,5].

In order to prevent the formation of blood clots within blood vessels, the interior of the free graft is cleansed with a saline solution or an organ-maintaining solution. To enhance the anticoagulant effect of these solutions, heparin is often added to these solutions [5].

In the interest of elevating the viability of cells of the graft conduit, various types of solutions—autologous whole blood, histidine-tryptophan-ketoglutarate (HTK), or University of Wisconsin solution (UWS)—are used, or the graft conduit cells are stored at a temperature of  $<4^{\circ}\text{C}$  [5,6].

The ideal antispastic drug for CABG should have no effect on the hemodynamics of the entire body, should suppress the contraction of blood vessels postoperatively, and should minimize apoptosis of the vascular cells of the graft conduit [7]. Therefore, a solution that is used only in the affected region is useful because it does not have any effect on the hemodynamics of the entire body and can easily be employed during an operation. Various drugs—such as nitroglycerin, nifedipine, verapamil, levosimendan, phenoxybenzamine, and papaverine—have been used for minimizing the spasms of blood vessels—graft conduit radial coronary artery, gastroepiploic artery, and great saphenous vein—that are used in the CABG procedure [7-11]. Of the antispastic drugs, papaverine is often used because of its powerful vasodilation effect on the region of interest.

When performing CABG, a dilute papaverine solution is either directly or indirectly applied on the exterior of the graft. Papaverine is widely used as a preservative agent during the CABG procedure because of its short-lived effect and its ability to maximally diminish the resistibility of the coronary artery [8]. Furthermore, when papaverine is injected directly into the coronary artery, it is reported that papaverine causes very minimal changes in the heart rate and the blood vessel resistance of the entire body [12]. However, papaverine is reported to reduce the function of endothelial cells in the internal thoracic artery and to induce apoptosis of human endothelial cells and smooth muscle cells of blood vessels [13].

Nafamostat mesilate (NM), the main drug of the experiment, is a proteinase inhibitor and is known to exhibit an an-

ticoagulant effect when it acts on the blood coagulators IX, X, XIIa, and VIIa [14]. NM has the physicochemical characteristic of being quickly metabolized in the liver and blood, thereby having a short half-life of 5 to 8 minutes. Further, because of its low molecular weight of 539.6 M, NM is easily removed from the body via extracorporeal circulation [15]. Therefore, NM is used for improving the conditions of acute pancreatitis and for preventing blood clot formation in the extracorporeal circulation in patients suffering from disseminative blood vessel coagulation, hemorrhagic lesion, or hemorrhagic tendency [16,17]. NM has been used mainly for hemodialysis or continuous renal replacement therapy [17], but the recent trend revealed an increasing use of NM in heart surgery involving heart-and-lung machines or in extracorporeal membrane oxygenation equipment [18,19].

Further, NM has an anti-inflammatory effect and suppresses nitric oxide (NO) overexpression, apoptosis, and interleukin (IL)-6 and IL-8 [20,21]. An enzyme, kallikrein, acts on kininogen, a molecule that exists in the fractional layer of alpha-2 globulin, to separate bradykinin and kallidin. The separated kinins have a tendency to expand peripheral blood vessels to control the blood pressure, and they also induce inflammation. Further, kallikrein can convert plasminogen into plasmin; thus, it is capable of triggering the fibrinolysis effect [22]. NM is known to prevent these actions of kallikrein, which is a type of serine protease, and thereby, works as an anti-inflammatory or antifibrinolytic drug [14,23]. Ryu et al. [24] reported that because of the suppression of the kallikrein-kinin system, when used as a pretreatment, NM can reduce the postperfusion syndrome, which is a condition of a sudden drop of blood pressure seen in the reperfused graft liver after a liver transplant.

Because of its anticoagulant, anti-inflammatory, and antifibrinolytic effects, NM was believed to act as a preservative solution for the graft conduit. However, the vasodilator ability of NM, one of its most important effects, needs to be studied. Therefore, we hypothesized that NM may be useful as a preserving agent for the graft conduit. Various tests—contractile, relaxation, and viability of vascular endothelial cells—were performed on NM and papaverine concomitantly to carry out a comparative experiment. Because NM is known to have an anti-inflammatory effect, apoptosis-reducing

function, and anticoagulation effect, we hypothesized that NM will be useful as a preserving agent for the graft conduit if it also has vasodilation action. For the investigation of NM as a conduit preservation solution, we performed *in vitro* and *in vivo* experiments as follows.

## METHODS

### 1) Experimental animals

Upon approval from the Chungnam National University Animal Experiment Committee Board, the appropriate experimental animals were carefully chosen according to the laboratory standards. The experiment was carried out with approximately 6-week-old and about 150 to 170-g Sprague-Dawley (SD) male rats. All the rats began fasting 12 hours before the experiment. No significant problem was observed in any of the animals.

### 2) Tension test of thoracic aorta of white mice

**(1) Tissue collections and organ bath:** The SD rats (n=6) were anesthetized by injecting 80 mg/kg of ketamine and 12 mg/kg of xylazine intraperitoneally. Thoracotomy was performed, 300 U/mL of heparin was injected into the left ventricle to prevent blood clot formation, and then the thoracic aorta was carefully extracted. The extracted thoracic aorta was placed in a laboratory dish containing the modified Krebs-Henseleit bicarbonate buffer solution at 4°C. The fat and connective tissue around the aorta was removed using stereomicroscopy. Taking care not to damage the vascular endothelial cells, the descending thoracic aorta was cleanly trimmed and cut into 5-mm-long aorta segments. Both ends of the excised aorta segments were hung on the stainless loops of an isometric force transducer (MultiMyograph 610 M; Danish Myo Technology, Copenhagen, Denmark), and they were installed in 7.5 mL of the modified Krebs-Henseleit bicarbonate buffer solution tank. By mixing 100 mM of NaCl, 4.7 mM of KCl, 1.9 mM of CaCl<sub>2</sub>, 1.2 mM of MgSO<sub>4</sub>, 1.03 mM of HK<sub>2</sub>PO<sub>4</sub>, 23 mM of NaHCO<sub>3</sub>, and 11.1 mM of glucose, we prepared the modified Krebs-Henseleit bicarbonate buffer solution. The buffer solution was placed in the organ bath and was maintained at 37°C, supplied with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and kept at a pH of 7.4. Tension was

applied gradually to the excised aortic vessel installed on the isometric force transducer. When the stabilized tension level was sustained at 2 g, the buffer solution was exchanged at 15-minute intervals for 60 minutes, and then, the equilibrium state was maintained. Using the method above, which is used for investigating the soundness of vascular endothelial cells of aorta segments, we examined the contraction reaction of the thoracic aorta using a high concentration of 60 mM of K<sup>+</sup>. Then, the results were used to revise the numerical value of the contraction and relaxation measurements.

**(2) Contraction and relaxation of the thoracic aorta:** In the interest of checking the contractile ability of the thoracic aorta, the buffer solution was exchanged three times in 1 hour and was stabilized. Phenylephrine (PE) was administered incrementally into the buffer solution, thereby raising the concentration level of PE from 10<sup>-8</sup> M to 3×10<sup>-7</sup> M and leading to an incremental increase of PE-induced contractions of the thoracic aorta. The degree of contraction was measured at the maximum level, and the contraction level achieved an equilibrium state. After the submaximally contracted aorta segments using PE reached the climax, acetylcholine (ACh) was administered incrementally and its concentration was increased from 10<sup>-8</sup> M to 10<sup>-5</sup> M. This was done to record the relaxation reaction of the aorta. The degree of contractile and relaxation reactions displayed on the polygraph in the unit of tension was measured. The relaxation reaction was expressed as a percentage according to the numerical submaximal contraction value of PE. This PE value was revised into the numerical submaximal contraction value induced via KCl.

**(3) Contraction and relaxation of papaverine and nafamostat mesilate:** After checking the contractile and relaxation abilities of the thoracic aorta, the buffer solution was exchanged three times to regain a stable status. Then, 3 µg/mL of papaverine and 10 µg/mL of NM were used as a pretreatment for 20 minutes. After continuously exchanging the buffer solution three times to remove the drugs, PE was administered incrementally by increasing its concentration from 10<sup>-8</sup> M to 3×10<sup>-7</sup> M in order to induce contraction in the aorta. The maximum contraction reaction point occurrence was observed, and the equilibrium point was attained. When the submaximally contracted aortic ring using PE reached the climax point, a relaxation reaction was recorded after increas-

ing the concentration of ACh incrementally from  $10^{-8}$  M to  $10^{-5}$  M. The degree of the contractile and relaxation reactions displayed on the polygraph in the unit of tension was measured. The relaxation reaction was expressed as a percentage according to the numerical submaximal contraction value of PE. This PE value was revised into the numerical submaximal contraction value induced via KCl.

### 3) Examination of the viability of vascular endothelial cells

**(1) Cytotoxicity test of vascular endothelial cells:** After  $1 \times 10^5$  human umbilical vein endothelial cells (HUVECs) were sprayed onto the 12 well plates, they were stabilized for 24 hours. NM and papaverine with increasing concentrations from—100 ng/mL to 100  $\mu$ g/mL, increasing in multiples of 10—were administered to the HUVECs in the order of the different concentration levels, and the cells were cultivated for an additional 24 hours. An automatic cell counter (ADAM-MC; Digital Bio, Seoul, Korea) was used for conducting the cytotoxicity test, and both live and dead cells were quantified after adding propidium iodide (PI). PI is known to couple with double-stranded nucleic acids. In our experiment, PI did not couple with the double-stranded nucleic acids of the live cells; however, it coupled with those of the dead or dying cells. Each well plate was sorted according to its concentration level, and the ratio between the number of cells that coupled with PI and the total number of cells was expressed as a percentage.

### 4) Histopathology and immunopathology tests on the thoracic aorta of white mice

**(1) Collection of tissue culture:** Approximately 6-week-old and about 150 to 170-g SD male rats (n=6) were anesthetized by injecting 4 mL/kg of 25% urethane into the abdominal cavity. They were put to death by dislocating their cervical vertebrae, and the thoracic aorta was extracted immediately after death. Then, the thoracic aorta was cut into 2-cm-long segments and immersed in a saline solution, NM solution (10  $\mu$ g/mL), or papaverine solution (10  $\mu$ g/mL) for 1 hour. Then, each segment was fixed in a 10% formalin solution for three days, and histopathology and immunopathology tests were carried out simultaneously.

**(2) Hematoxylin and eosin stain:** The thoracic aorta segments, which were fixed in 10% formalin after the extraction, were cut along the cross-section line of the thoracic aorta into 4-mm-thick segments. Microscope slides were prepared for each segment. A paraffin-embedded block was made and sliced with 5  $\mu$ m. Thereafter, the hematoxylin and eosin (H&E) stain was performed.

**(3) Pathological assessment:** One pathologist, who was not aware of the content of the experimental groups, carried out the pathological assessment for all cases. The pathologist observed the nuclei and the cytoplasm of the cells of the intima and media membranes, searched for any structural damage, and studied the overall morphology of the cells. The images of the blood vessel fragment dyed with H&E staining were scanned and transferred to a computer, and a quantitative morphometric analysis was performed using the Scion Image Analysis software (ver. 1.0). We evaluated the thickness of the intima and media membranes. The thickness of the media membrane was measured at eight places that were 45° apart and were located around the central site of the blood vessel as a reference point. The results were averaged at each point to compute the thickness of the media membrane.

**(4) Terminal deoxynucleotidyl transferase dUTP nick end labeling assay:** Of the slides that were prepared in a different type of solutions, one slide from each group was taken to perform a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the ApopTag peroxidase kit (peroxidase in situ apoptosis detection kit Chemicon #S7100; North Ryde, NSW, Australia).

The paraffin on the tissue slides, which were prepared with 4- $\mu$ m-thick paraffin, was removed with 100% xylene. Then, the slides were treated with 100%, 95%, and 70% alcohol and washed with distilled water. Next, the slides were immersed in a phosphate buffered saline+Tween 20 (PBST) solution for 10 minutes and were allowed to react with 1:200 diluted proteinase K at room temperature for 30 minutes. The slides were then washed twice with distilled water for 2 minutes each and were reacted with 3%  $H_2O_2$  for 5 minutes to suspend non-specific reactions of endogenous peroxidase (quench endogenous peroxidase). Then, they were washed with PBST for 1 minute. Next, the slides were treated with a

labeling buffer for 5 minutes. The 50  $\mu\text{L}$  of the labeling reaction mixture (1- $\mu\text{L}$  TdT dNTP+1- $\mu\text{L}$  bivalent cation ( $\text{Mn}^{++}$ )+1- $\mu\text{L}$  TdT enzyme+50- $\mu\text{L}$  labeling buffer) was dropped on the slides and covered with a parafilm. The slides were then allowed to rest overnight in a humidity-controlled room at 4°C. Thereafter, they were treated with a stop buffer for 5 minutes and washed twice with PBST for 2 minutes each time. Next, the slides were allowed to react with 50  $\mu\text{L}$  of streptavidin at room temperature for 25 minutes and washed twice with PBST for 2 minutes each time. A diaminobenzidine solution was used for 3 minutes to create color on the slides; then, these slides were washed with distilled water. Methyl green was used for 10 minutes to counter-stain the slides; the slides were then washed briefly three times. Thereafter, the slides were immersed in 100% n-butanol twice for 2 minutes each time, dried in xylene four times for 2 minutes each time, and then covered with cover slides.

All the steps were carried out in a humidity-controlled room. The slides were dried completely, fixed on a microscope, and studied at room temperature. Cells with nuclei that were dyed brown were judged to be TUNEL-positive cells that underwent apoptosis.

### 5) Statistical process

PASW SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA) was used for analyzing the data and processing the statistics; continuous variables were expressed as average $\pm$ standard deviation, and categorical variables were expressed as median values. An analyses of variance (ANOVA) test was conducted to compare the continuous variables across the groups. A one-way ANOVA test was used for examining the histopathology test, and a Mann-Whitney U test was employed to evaluate the immunopathology test. Any results of interest were examined using Tukey's multiple comparison test. If the p-value was less than 0.05, then the data were regarded as statistically significant.

## RESULTS

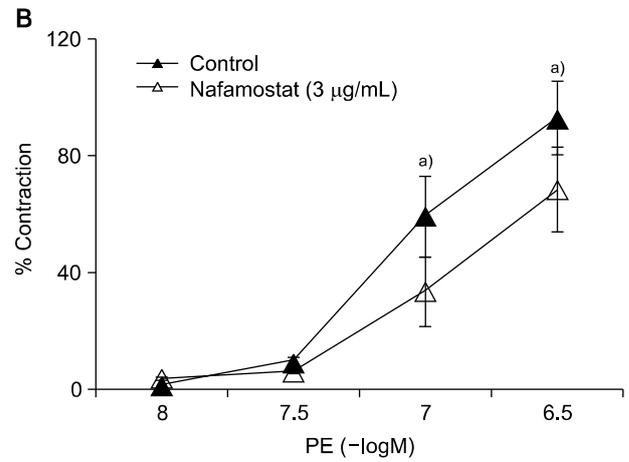
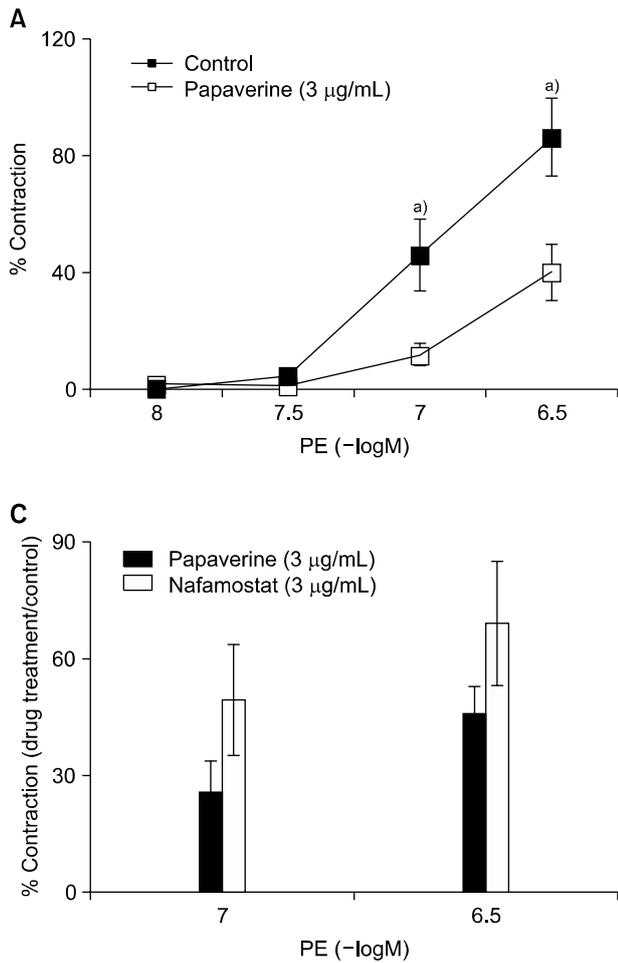
### 1) Results of tension test

**(1) Results of contractile test on papaverine and nafamostat mesilate:** The normal control group of aorta segments

was pretreated with either 3  $\mu\text{g}/\text{mL}$  of papaverine or 3  $\mu\text{g}/\text{mL}$  of NM, and then, the results of the contractile reaction of both groups to the PE were studied. Both groups began showing a significant contractile reaction when the PE concentration reached  $10^{-7}$  M. At  $10^{-7}$  M of PE, as compared to the control groups, the papaverine-treated group displayed 14.3% and the NM-treated group exhibited a 40.1% contractile effect ( $p < 0.05$ ). At  $3 \times 10^{-7}$  M of PE, as compared to the control groups, the papaverine-treated group showed a 26.9% contractile effect and the NM-treated group exhibited a 68.6% contractile effect ( $p < 0.05$ ). Although the papaverine group seemed to have a better diminishing effect on the contractile ability than the NM group, it did not show any significant difference, from a statistical perspective, at the two different concentrations of PE ( $p > 0.05$ ) (Fig. 1).

The normal control group of aorta segments was pretreated with either 10  $\mu\text{g}/\text{mL}$  of papaverine or 10  $\mu\text{g}/\text{mL}$  of NM, and then, the results of the contractile reaction of both groups to PE were studied. The NM-treated group began showing a definite contraction at  $3 \times 10^{-8}$  M of PE, whereas the papaverine-treated group started displaying a contraction at  $3 \times 10^{-7}$  M of PE. At  $3 \times 10^{-8}$  M of PE, as compared to the control groups, the papaverine-treated group exhibited a 12.5% contractile effect and the NM-treated group presented an 83.9% contractile effect ( $p < 0.05$ ). At  $10^{-7}$  M of PE, as compared to the control groups, the papaverine-treated group showed a 2.7% contractile effect and the NM-treated group displayed a 39.2% contractile effect ( $p < 0.05$ ). At  $3 \times 10^{-7}$  M of PE, as compared to the control groups, the papaverine-treated group exhibited a 7.0% contractile effect and the NM-treated group presented a 58.4% contractile effect ( $p < 0.05$ ). At every point in the concentration range of PE from  $3 \times 10^{-8}$  M to  $3 \times 10^{-7}$  M, papaverine showed, from a statistical point of view, a significantly different diminishing effect in the contractile ability from that of NM ( $p < 0.05$ ) (Fig. 2).

**(2) Results of relaxation test on papaverine and nafamostat mesilate:** The normal control group of aorta segments was pretreated with either 3  $\mu\text{g}/\text{mL}$  of papaverine or 3  $\mu\text{g}/\text{mL}$  of NM, and then, the degree of contractile reaction to the PE was increased to that of the concentration of  $3 \times 10^{-7}$  M of PE to induce the submaximal contraction of the blood vessel. Next, ACh was applied gradationally starting from



**Fig. 1.** Cumulative contraction–response curves for contractile responses to phenylephrine (PE) in the control solution, (A) 3 µg/mL of papaverine, and (B) 3 µg/mL of nafamostat mesilate. Contractile responses to PE  $10^{-7}$  M and  $3 \times 10^{-7}$  M in (A) 3 µg/mL of papaverine and (B) 3 µg/mL of nafamostat mesilate were statistically decreased as compared to those of the control. (C) The results of contraction with papaverine and nafamostat mesilate are expressed as a percentage as compared to that of the control at the same PE concentration. The percentage contraction observed in the cases of papaverine and nafamostat mesilate was not significantly different in  $10^{-7}$  M and  $3 \times 10^{-7}$  M of PE. <sup>a)</sup> $p < 0.05$ .

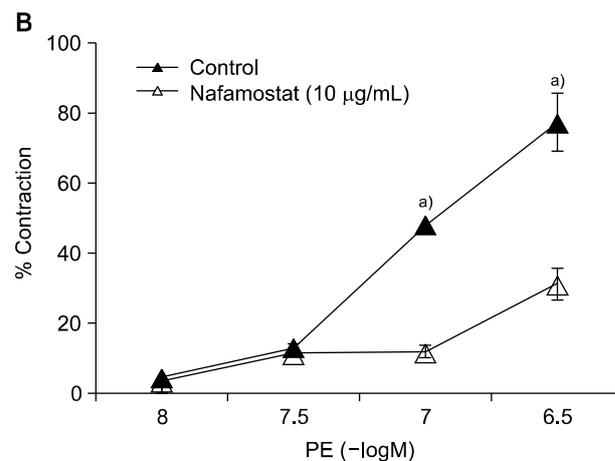
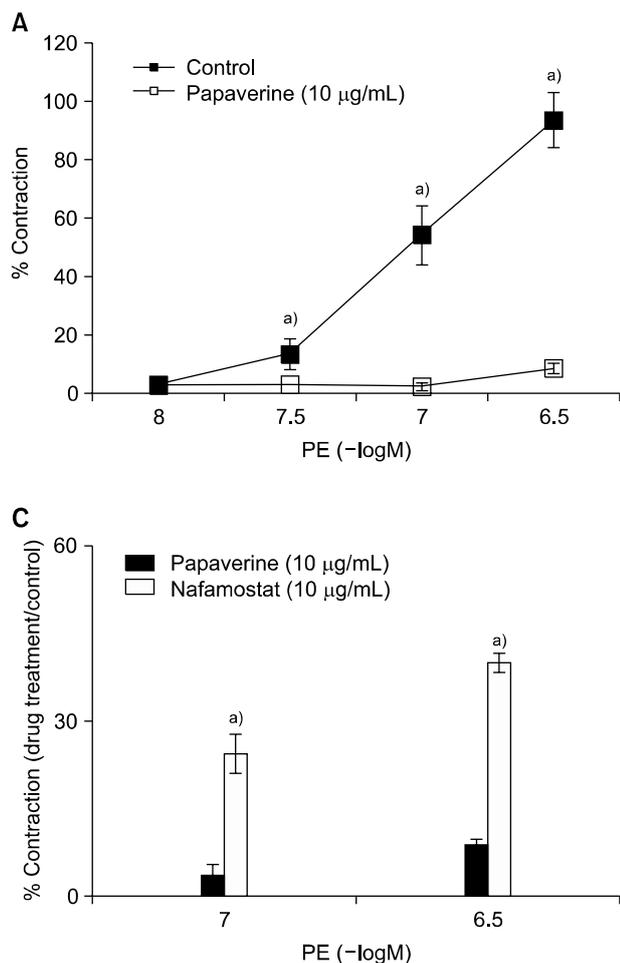
$10^{-9}$  M to  $10^{-5}$  M to both the experimental groups, and the results of the relaxation reaction of the aorta were studied. Both groups started showing significant relaxation results at  $10^{-8}$  M of ACh. At  $10^{-8}$  M of ACh, the papaverine-treated group showed 60.9% and the NM-treated group displayed a 76.5% relaxation effect. At  $10^{-7}$  M of ACh, the papaverine-treated group exhibited a 23.4% relaxation effect and the NM-treated group displayed a 32.0% relaxation effect. At  $10^{-6}$  M of ACh, the papaverine-treated group showed a 24.9% relaxation effect and the NM-treated group displayed a 22.5% relaxation effect. At  $10^{-5}$  M of ACh, the papaverine-treated group exhibited an 18.0% relaxation effect and the NM-treated group presented a 17.5% relaxation effect (Table 1). Although the papaverine group seemed to have better relaxation ability than the NM group, it did not show any statistically significant difference at any of the four different con-

centrations of ACh ( $p > 0.05$ ) (Fig. 3).

The normal control group of aorta segments was pretreated with either 10 µg/mL of papaverine or 10 µg/mL of NM, and then the blood vessel was treated with PE to induce its submaximal contraction level. However, as compared to the normal control group, the papaverine-treated group showed an insignificant contraction of only 2.7%. Therefore, the relaxation reaction change in the case of ACh with respect to the submaximal contraction of the papaverine-treated group did not show any meaningful results. These results were not analyzed.

## 2) Results of viability test on human umbilical vein endothelial cells

HUVECs were administered at different concentration levels of NM, ranging from 100 ng/mL to 100 µg/mL in incre-



**Fig. 2.** Cumulative contraction-response curves for the contractile responses to phenylephrine (PE) in the control, (A) 10-µg/mL papaverine, and (B) 10-µg/mL nafamostat mesilate. (A) Contractile responses to  $3 \times 10^{-8}$ -M,  $10^{-7}$ -M, and  $3 \times 10^{-7}$ -M PE in 10-µg/mL papaverine showed a statistically significant decrease as compared to those of the control. (B) Contractile responses to  $10^{-7}$ -M and  $3 \times 10^{-7}$ -M PE in 10-µg/mL nafamostat mesilate also showed a statistically significant decrease as compared to those of the control. (C) The results of contraction with papaverine and nafamostat mesilate are expressed as a percentage as compared to those of the control at the same PE concentration. There are significant differences between the contraction percentage of papaverine and nafamostat mesilate in  $10^{-7}$ -M and  $3 \times 10^{-7}$ -M PE. <sup>a</sup> $p < 0.05$ .

**Table 1.** Results of relaxation test with acetylcholine were obtained after preincubation with papaverine and nafamostat mesilate

Drug	Mole				
	$10^{-9}$	$10^{-8}$	$10^{-7}$	$10^{-6}$	$10^{-5}$
Papaverine (%)	100	60.9	23.4	24.9	18.0
Nafamostat mesilate (%)	100	76.5	32.0	22.5	17.5

ments of ten-fold. The results after cultivating the cells for 24 hours showed that the control group displayed 100% viability in the case of 100 ng/mL to 10 µg/mL of NM and 97% viability in the case of 1 µg/mL to 100 µg/mL of NM (Table 2, Fig. 4).

HUVECs were administered at different concentration levels of papaverine ranging from 100 ng/mL to 100 µg/mL in increments of ten-fold. The results after cultivating the cells for 24 h showed that the control group showed 98% viability

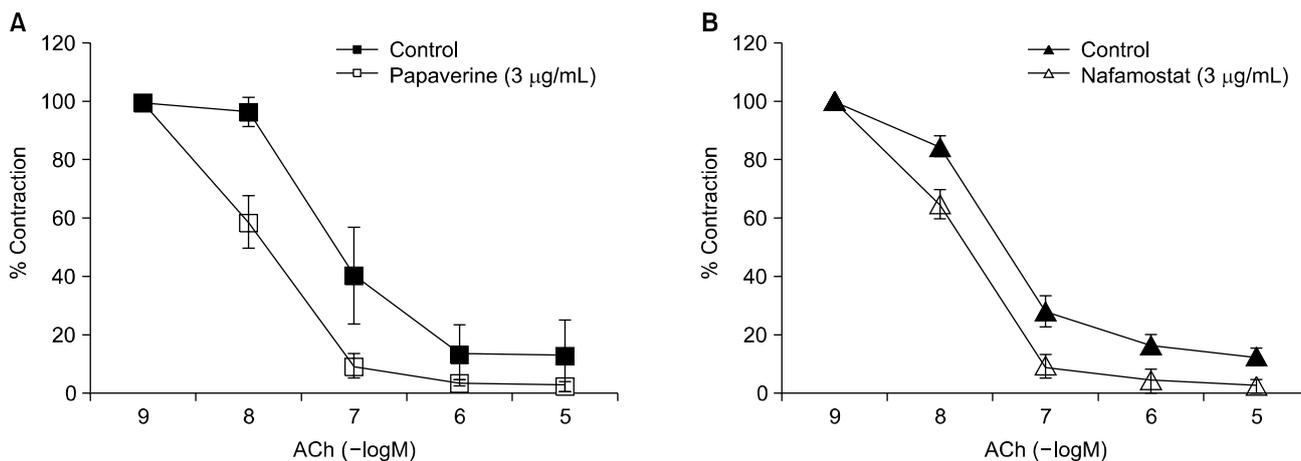
in 100 ng/mL of papaverine, 96% viability in 1 µg/mL of papaverine, and 95% and 91% viability in 10 ng/mL and 100 µg/mL of papaverine, respectively (Table 2, Fig. 5).

### 3) Results of histopathologic and immunohistologic tests on thoracic aorta of Sprague-Dawley rat

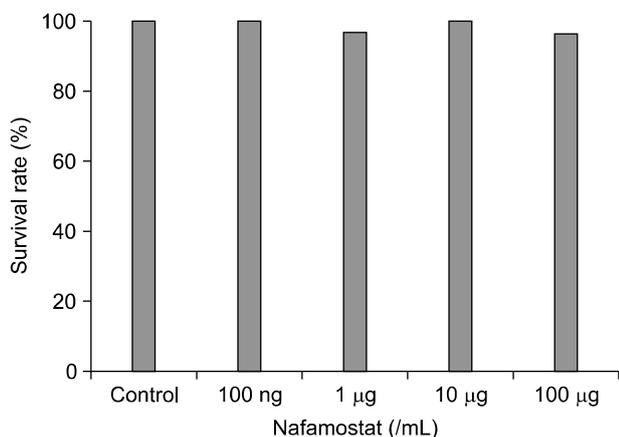
**(1) Results of hematoxylin and eosin stain intima and media membrane thickness comparison:** In the H&E stain, the structural damage and the overall morphology of the nu-

**Table 2.** Results of human umbilical vein endothelial cell culture of the rat thoracic aorta following preincubation with saline, papaverine (0.1, 1, 10, and 100  $\mu\text{g/mL}$ ), and nafamostat mesilate (0.1, 1, 10, and 100  $\mu\text{g/mL}$ )

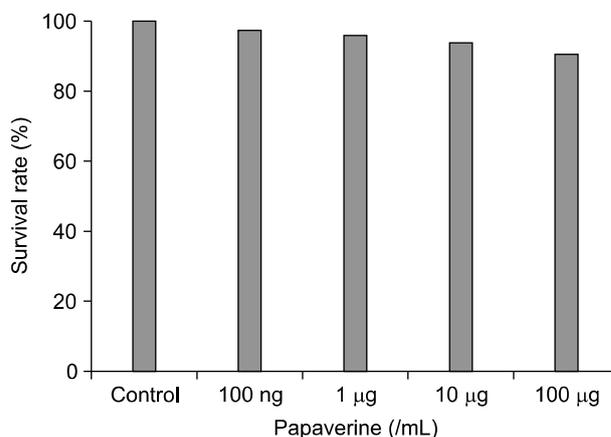
Drug	Concentration				
	Control	0.1 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$	10 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$
Nafamostat mesilate (%)	100	100	100	97	97
Papaverine (%)	100	98	96	95	91



**Fig. 3.** Cumulative relaxation-response curves for the relaxation responses to acetylcholine (ACh) for the control, (A) 3  $\mu\text{g/mL}$  of papaverine, and (B) 3  $\mu\text{g/mL}$  of nafamostat mesilate. Relaxation responses to each ACh concentration in 3  $\mu\text{g/mL}$  of papaverine and 3  $\mu\text{g/mL}$  of nafamostat mesilate were not statistically different compared with those of the control.



**Fig. 4.** Results of human umbilical vein endothelial cell viability following treatment with varying concentrations of nafamostat mesilate (range, 100 ng/mL to 100  $\mu\text{g/mL}$ ). The results are expressed as percentage viability.



**Fig. 5.** Results of human umbilical vein endothelial cell viability following treatment with each papaverine concentration (range, 100 ng/mL to 100  $\mu\text{g/mL}$ ). The results are expressed as percentage viability.

clei and the cytoplasm of intima and media membrane cells were studied. Further, thicknesses of the intima and media membranes were measured. The measurements of the intima

membrane thickness were as follows:  $5.03 \pm 1.08 \mu\text{m}$  for the control group,  $5.16 \pm 1.26 \mu\text{m}$  for the papaverine-treated group, and  $4.87 \pm 1.13 \mu\text{m}$  for the NM-treated group. There was no

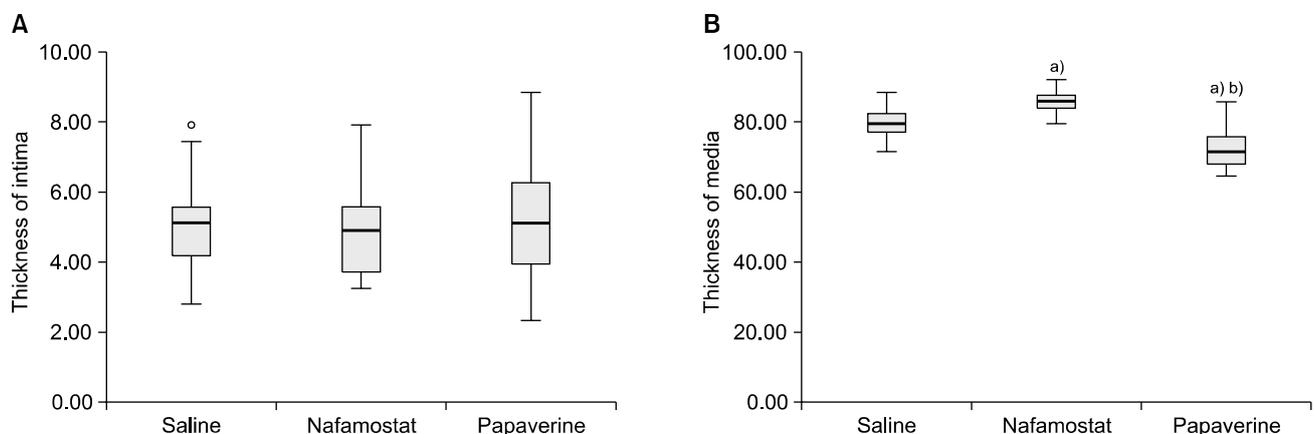
**Table 3.** Quantitative histopathological analysis of the rat thoracic aorta thickness following preincubation with saline, papaverine (10 µg/mL), and nafamostat mesilate (10 µg/mL)

	Group (n=48)			p-value <sup>a)</sup>
	Saline	Nafamostat mesilate	Papaverine	
T <sub>intima</sub> (µm)	5.03±1.08	4.87±1.13	5.16±1.26	0.575
T <sub>media</sub> (µm)	79.43±4.27	85.98±2.93 <sup>b)</sup>	72.35±5.46 <sup>c)</sup>	0.000
T <sub>intima+media</sub> (µm)	54.46±4.32	90.85±2.69 <sup>b)</sup>	78.86±5.47 <sup>c)</sup>	0.000

T<sub>intima</sub>, thickness of intima; T<sub>media</sub>, thickness of media; T<sub>intima+media</sub>, thickness of intima and media.

<sup>a)</sup>Statistical significances were tested by one-way analysis of variances among groups.

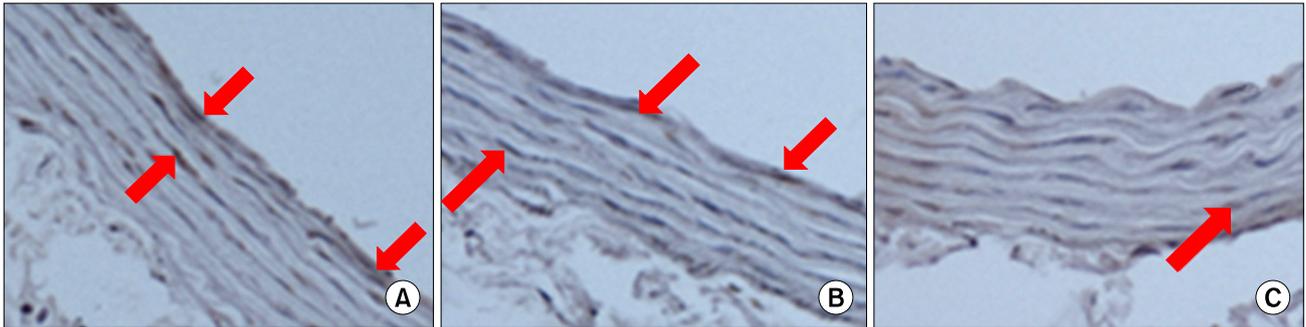
<sup>b)</sup> and <sup>c)</sup> indicate the significant difference between groups on the basis of Tukey's multiple comparison test.



**Fig. 6.** Histopathological analysis of the rat thoracic aorta thickness following preincubation with saline (control), papaverine (10 µg/mL), and nafamostat mesilate (10 µg/mL). (A) No statistically significant difference was observed between the intimal thickness of the control, papaverine-treated, and nafamostat mesilate-treated groups. (B) There are statistically significant differences between the medial thickness of the control, papaverine-treated, and nafamostat mesilate-treated groups. <sup>a)</sup> $p < 0.05$  compared with the control group (Mann-Whitney U test). <sup>b)</sup> $p < 0.05$  compared with the nafamostat mesilate-treated group (Mann-Whitney U test). Box boundaries: 25th to 75th percentile. Thick line within the box: median. Whiskers above and below the box: 10th to 90th percentile. Open circle: full range of data.

statistically significant difference among the results of the intima thicknesses. The measurements of the media membrane thickness were 79.43±4.27 µm for the control group and 72.35±5.46 µm for the papaverine-treated group. The NM group showed a greater numerical value for the measurement of the media membrane thickness of 85.98±2.93 µm, for it had the lowest degree of cytoplasm morphology destruction of the three groups ( $p < 0.05$ ) (Table 3). The papaverine-treated group displayed the smallest numerical value for the measurement of the media membrane thickness, and this was statistically significant ( $p < 0.05$ ) (Fig. 6). This concluded because there was the highest degree of cytoplasm morphology destruction in the papaverine-treated group.

**(2) Results of terminal deoxynucleotidyl transferase dUTP nick end labeling assay:** The nuclei in the TUNEL-positive cells that were dyed brown because of the progress of apoptosis were observed. With respect to the NM-treated group, a majority of the nuclei of the cells in the control and papaverine-treated groups were dyed brown, and thus, they were TUNEL-positive (Fig. 7). The least amount of approximately 29% of the intima and media membrane cells in the NM-treated group was discovered to be TUNEL-positive. Eighty percent and 74% TUNEL-positive cells were observed in the control group and the papaverine-treated group, respectively, indicating a more advanced progression of the apoptosis of the intima and media membrane cells.



**Fig. 7.** Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of the rat thoracic aorta following preincubation with (A) saline, (B) papaverine (10 µg/mL), and (C) nafamostat mesilate (10 µg/mL). TUNEL-positive cells were noted more frequently in the control and the papaverine-treated groups (×400). Red arrow: TUNEL-positive cell.

## DISCUSSION

In the interest of improving the patency of the graft conduit in CABG, various types of experiments have been performed. In order to increase the viability of the cells of the graft conduit, various types of solutions—autologous whole blood, HTK, or UWS—are used or the graft conduit cells are stored at a temperature of <math>4^{\circ}\text{C}</math> [5,6]. After studying a pig vein, which was stored for 24 hours in either the blood of the pig at  $4^{\circ}\text{C}$  or a saline solution, using a scanning microscope, Cavallari et al. [25] argued that storing a blood vessel in the blood of the specimen has no advantage over keeping it in a saline solution. Santoli et al. [6] claimed that because UWS has adenosine-like components, it is more effective in increasing the viability of the cells of the graft conduit.

In order to prevent the formation of blood clots within blood vessels, the interior of the free graft is cleansed with a saline solution or an organ-maintaining solution. To enhance the anticoagulant effect of these solutions, heparin is often added to the solution [5]. The action of antithrombin is accelerated by 1,000 times under the existence of heparin; thus, the effectiveness of the anticoagulation mechanism of heparin increases rapidly.

Papaverine is widely used as a powerful vasodilator in the affected region during CABG because of its short-lived effect and its ability to maximally diminish the resistibility of the coronary artery [8]. However, papaverine is reported to reduce the function of endotheliocytes in the internal thoracic

artery and to induce apoptosis of human endotheliocytes and smooth muscle cells of blood vessels [13]. Another study reports that because papaverine itself is acidic, it is a potential factor of vascular endothelial injury [26].

NM (6-amidino-2-naphthyl-4-guanidinobenzoate-dimethanesulfonate: FUT-175, Futhan)—researched for its role as a preservative solution for the graft conduit during CABG—is known to exhibit an anticoagulant effect when it acts on the blood coagulators IX, X, XIIa, and VIIa [14]. Further, NM has an anti-inflammatory effect and suppresses NO over-expression, apoptosis, IL-6, and IL-8 [20,21]. NM also acts as a kallikrein inhibitor, a type of serine protease, thereby known to work as an anti-inflammatory or antifibrinolytic drug [14,23].

In this study, the vascular contractile reaction to PE under conditions of 3 µg/mL of papaverine or 3 µg/mL of NM did not show any significant difference, although the papaverine-treated group seemed to have a better diminishing effect on the contractile ability than the NM-treated group (Fig. 1). In the same test performed under the conditions of 10 µg/mL of papaverine or 10 µg/mL of NM, papaverine showed, from a statistical perspective, a significantly different diminishing effect of contractile ability than NM (Fig. 2). The results of the vascular relaxation reaction—acquired after pretreating the blood vessel of interest with either 3 µg/mL of papaverine or 3 µg/mL of NM—indicated that although the papaverine-treated group seemed to have a better relaxation ability than the NM-treated group, it did not show any significant difference, at four different concentrations of ACh (Fig. 3).

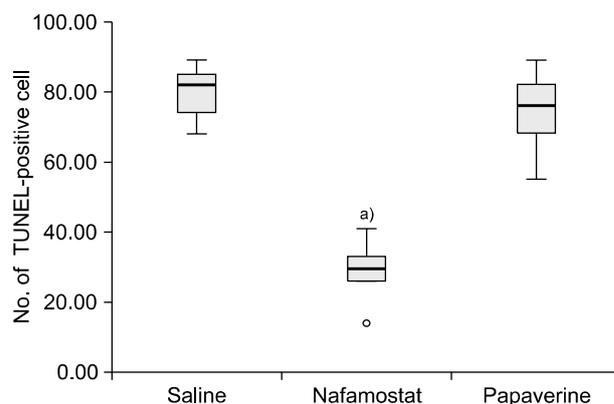
Overall, these results suggested that NM displayed similar vascular relaxation ability to that of papaverine at a concentration of 3  $\mu\text{g/mL}$ , but papaverine exhibited better vascular relaxation ability at a concentration of 10  $\mu\text{g/mL}$ .

Papaverine is an unspecific vasodilator that works through various mechanisms, but in general, it acts as a phosphodiesterase inhibitor and increases the cyclic guanosine monophosphate concentration within the smooth vascular muscle cells [27]. Further, papaverine accomplishes its vasorelaxation role by suppressing the calcium influx or restricting the calcium secretion from a cell's storage [28]. However, thus far, there have been no reports on either the vasorelaxation effect of NM or the mechanism of its vasodilating action; therefore, additional research on these topics is necessary.

In the viability test of vascular endothelial cells conducted by cultivating HUVECs, the NM-treated group showed excellent viability of >97% at four different concentration levels (Fig. 4) and the papaverine-treated group displayed a viability of 91% at four different concentration levels (Fig. 5). Therefore, the results of the viability testing of vascular endothelial cells indicated that both drugs exhibited excellent viability.

The histopathological test conducted to investigate the protective effect of NM against the apoptosis of vascular cells indicated that the nuclei and the cytoplasm of the vascular endothelial cells and the vascular media membrane were most well preserved in the NM-treated group. The vascular intima membrane thickness did not show any significant difference among the control, NM-treated, or papaverine-treated groups; however, the vascular media membrane thickness was noticeably different. The vascular media membrane thicknesses in decreasing order were as follows: NM-treated group, control group, and papaverine-treated group (Fig. 6).

The results of the TUNEL assay showed that 29% TUNEL-positive cells were found in the NM-treated group, but 80% and 74% of TUNEL-positive cells were found in the control and papaverine-treated groups, respectively, due to the more advanced apoptosis (Fig. 8). Because endothelial cells are organized in a single layer, the vascular intima membrane thickness did not show any statistical difference even in the presence of a considerable amount of apoptosis. However, the vascular media membrane is composed of multiple layers,



**Fig. 8.** Total number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells of the rat thoracic aorta following preincubation with saline, papaverine (10  $\mu\text{g/mL}$ ), and nafamostat mesilate (10  $\mu\text{g/mL}$ ). The results of the TUNEL assay show the number of TUNEL-positive cells of the nafamostat mesilate-treated group is statistically significant lower than that of the control and papaverine-treated groups. <sup>a)</sup> $p < 0.05$  compared with the control and the papaverine-treated group (Mann-Whitney U test). Box boundaries: 25th to 75th percentile. Thick line within the box: median. Whiskers above and below the box: 10th to 90th percentile. Open circle: full range of data.

thus showing a statistical difference in the vascular media membrane thickness when the cytoplasm was destroyed due to apoptosis.

Overall, the NM-treated group displayed a better protective effect against apoptosis. This result agrees with a research that reported that papaverine may induce apoptosis in endothelial cells and smooth vascular muscle cells in the human internal thoracic artery [13]. Further, NM suppresses NO overexpression, apoptosis, and the formation of IL-6 and IL-8 [21,22]. NM also hinders the production of superoxide, hydrogen peroxide, and hydroxyl radicals from human polymorphonuclear leukocytes [29,30]. The results of the endothelial cell viability test and apoptosis in the vascular cell test coincide with those of previous scientific papers that discussed the anti-inflammatory and the protective ability against apoptosis in the vascular endothelial cells of NM.

In conclusion, the conclusions of the research that investigated the effectiveness of papaverine and NM as a preservative solution for a coronary artery conduit were as follows. First of all, at a concentration of 3  $\mu\text{g/mL}$ , NM exhibited a similar vasorelaxation effect as papaverine. Second, the via-

bility of the vascular endothelial cells and the protective ability against apoptosis in the vascular cells were both outstanding in the NM-treated group. Further, NM was shown to be a drug that could likely be used a preservative solution in CABG surgery because it can block hematoma formation due to its anticoagulation and antifibrinolytic actions. Moreover, the drug can decrease hemorrhage in the sutured region.

A limitation of the research is that only the aorta of white mice was used and human tissue was not the subject of the experiment. Future research will utilize blood vessels that are used in actual CABG—surgery the internal thoracic artery, radial artery, and great saphenous vein—to study whether similar results can be observed in humans. Further, a study on the injection method and the appropriate volume that are suitable for humans and a study on the vasorelaxation mechanism of NM from a molecular biological point of view are necessary.

## CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

## ACKNOWLEDGMENTS

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